Published in final edited form as:

Biochemistry. 2011 May 3; 50(17): 3405–3407. doi:10.1021/bi200266v.

S-Nitrosylation of ApoE in Alzheimer's Disease

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Abstract

The mechanism by which apolipoprotein E (ApoE) isoforms functionally influence the risk and progression of late-onset Alzheimer's disease (LOAD) remains hitherto unknown. Herein, we present evidence that all ApoE isoforms bind to nitric oxide synthase 1 (NOS1) and that such protein-protein interaction results in *S*-nitrosylation of ApoE2 and ApoE3 but not ApoE4. Our structural analysis at atomic level reveals that *S*-nitrosylation of ApoE2 and ApoE3 proteins may lead to conformational changes resulting in the loss of binding to low-density lipoprotein (LDL) receptors. Collectively, our data suggest that *S*-nitrosylation of ApoE proteins may play an important role in regulating lipid metabolism and in the pathogenesis of LOAD.

The etiology of LOAD is strongly dependent upon various genetic and environmental factors and possibly further intertwined with complex gene-gene and gene-environment interactions. ApoE is the single most significant genetic risk factor identified for LOAD, the leading cause of dementia in the elderly, primarily through genetic mapping in the early 1990s (1–4). ApoE is expressed within human brain in three distinct isoforms, termed ApoE2, ApoE3 and ApoE4, which only differ in amino acids at positions 112 and 158. Thus, while ApoE2 contains two cysteine residues at positions 112 and 158, C158 is replaced by an arginine in ApoE3, and in ApoE4, both cysteine residues at positions 112 and 158 are replaced by arginine. Although ApoE4 has been universally confirmed as a risk gene for LOAD, many individuals affected by LOAD do not carry a single risk-conferring ApoE4 allele but, on the contrary, the most common risk-neutral ApoE3 allele. Importantly, the ApoE4 risk polymorphism is neither necessary nor sufficient for the onset of LOAD due to the fact that as much as 50% of the genetic-risk effect remains unexplained (5).

Since the identification of ApoE in LOAD, numerous studies have been conducted to uncover the functional role of various isoforms of ApoE. Differential biological effects among ApoE isoforms have been documented, implicating potential pathways of ApoE in LOAD pathogenesis (6–7). Additionally, it is not fully understood as to why the R112C amino acid substitution that converts ApoE4 to ApoE3 produces such dramatically different outputs in risk of LOAD. Cysteine residues play a critical part in driving protein folding, metal ion chelating, posttranslational modifications such as palmitoylation and prenylation, and thiol-based redox regulatory switches. Accordingly, the replacement of cysteine residues by arginine in ApoE3 and ApoE4 is likely to modulate the function of these isoforms relative to ApoE2. Due to the distinct effects of ApoE isoforms in LOAD risk, it is likely that the cysteine residues in ApoE2 and ApoE3 serve as on/off regulatory switches.

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SUPPORTING INFORMATION AVAILABLE

Detailed experimental methods and 3D atomic modeling are available free of charge via the Internet at <http://pubs.acs.org>.

Our hypothesis is that NOS1 catalyzes *S*-nitrosylation, a nitric oxide (NO) signal specified posttranslational modification, of C112 in ApoE3 within human brain and that the *S*-nitrosylated ApoE3 contributes to the risk of LOAD.

To test our hypothesis, we measured the interaction between ApoE proteins and NOS1 in a cell-based model. Briefly, we transiently transfected an NOS1 stably overexpressed HEK-293 cell line with each of the three ApoE isoform constructs. Co-immunoprecipitation (Co-IP) showed that all ApoE isoforms bound to NOS1 with no obvious differences among the Co-IP bands, suggesting that ApoE2, ApoE3 and ApoE4 proteins bind to NOS1 with similar affinities (Figure 1A). Furthermore, we also observed co-localization of endogenous ApoE3 and NOS1 in a homozygous ApoE3 human hippocampus using immunofluorescence (Figure 1B). Taken together, these data suggest strongly that ApoE proteins physically interact with NOS1.

The ApoE-NOS1 interaction suggests a possible regulatory mechanism. In ApoE transgenic mice, ApoE4 allele, but not ApoE2 and ApoE3 alleles, is associated with a higher NO production in microglial cells (8). This effect could possibly be attributed to the direct interaction between ApoE4 and NOS1, in addition to an increased arginine transport. A higher level of NO and excessive nitrosative and oxidative stress have long been suggested to play pivotal roles in the pathogenesis of neurodegenerative disorders including LOAD (9).

On the other hand, compelling evidence supports that a key determinant of the specificity in NO signal transduction is the interaction between NOS enzymes and other cellular proteins, which are targets of *S*-nitrosylation (10). *S*-nitrosylation, coupling of a NO moiety to a reactive cysteine thiol, has emerged as a ubiquitous protein posttranslational modification (11). In a manner akin to protein phosphorylation, *S*-nitrosylation switches the on/off functions of receptors, GTPases, transcription factors and other proteins. Interestingly, the only difference among various ApoE isoforms is the number of cysteine residues (two in ApoE2, one in ApoE3 and none ApoE4) that may serve as potential targets for *S*-nitrosylation.

To test whether ApoE proteins are subject to *S*-nitrosylation, we purified recombinant human ApoE proteins and subjected them to a biotin-switch assay (12). In the presence of an NO donor (100 μ M GSNO), bands were only observed for ApoE2 and ApoE3 but not for ApoE4 (Figure 2A). Importantly, the intensity of *S*-nitrosylated ApoE2 band is much stronger than the *S*-nitrosylated ApoE3, suggesting that both cysteine residues in ApoE2 are specifically targeted by NO.

Encouraged by this result, we next performed the biotin-switch assay on human hippocampus lysates from homozygous ApoE3 allele and homozygous ApoE4 allele tissue (ApoE2 homozygous human brain is rare and was not available for this study). All samples showed a band at ~60KD, suggesting an endogenous biotinylated protein. Consistent with the findings on recombinant purified ApoE proteins, only ApoE3 samples showed *S*-nitrosylated bands at ~35KD, while no *S*-nitrosylated bands for ApoE4 samples were observed (Figure 2B). Interestingly, in the control treatment (100 μ M GSH), ApoE3 samples also displayed *S*-nitrosylated band, suggesting endogenous *S*-nitrosylation of ApoE3 in human hippocampus. With the treatment of NO donor (100 μ M GSNO), ApoE3 *S*-nitrosylated band was much stronger, suggesting a dynamic *S*-nitrosylation paradigm for ApoE3 in the human brain.

To further understand the physiological significance of *S*-nitrosylation of ApoE proteins, we generated 3D atomic models of the WT and C112SNO *S*-nitrosothiol derivative of the receptor binding (RB) domain of ApoE2 and ApoE3 using the corresponding known crystal

structure as a template (Figure 3A) (13). Our modeled structures display the canonical fold comprised of an anti-parallel four-helical bundle ($\alpha 1$ - $\alpha 4$) capped at one end by an additional helix ($\alpha 1'$) arranged in a perpendicular fashion to the four-helical axis (Figures 3B–C). The helices within the helical bundle display strong amphipathic character in that the inner core of the bundle is largely enriched with hydrophobic residues while the solvent-exposed exterior is predominantly decorated with charged and hydrophilic residues. Accordingly, the stability of the four-helical bundle arises from a tightly packed network of van der Waals contacts within the inner hydrophobic core of the protein in a manner akin to hydrophobic interactions stabilizing leucine zippers and by virtue of an extensive network of intramolecular ion pairing between oppositely charged residues on the external protein surface. Additionally, it is believed that the charged residues not involved in intramolecular ion pairing on the surface of the protein play a central role in binding to LDL receptors by virtue of their ability to engage in intermolecular contacts via ion pairing.

Our 3D atomic models suggest that C112 lies in close proximity to charged residues R61 and E109 on the surface of the protein. Remarkably, R61 and E109 rank among some of the oppositely charged residues that are not involved in the stabilization of the four-helical bundle of the RB domain through the formation of intramolecular ion pairs. It is thus conceivable that R61 and E109 may be involved in mediating the binding of the RB domain to the LDL receptors. Indeed, the key role of R61 in LDL receptor binding is supported by experimental evidence (14). How could *S*-nitrosylation of C112 affect the binding of RB domain to LDL receptors? While the poorly polarizable thiol moiety of C112 is unlikely to participate in intramolecular contacts with the R61 and E109 neighbors, *S*-nitrosylation of C112 is likely to impart enhanced polarity on the resulting *S*-nitrosothiol (C112SNO) group. Additionally, the *S*-nitrosothiol group of C112SNO may undergo resonance arrangement to form a zwitterion with an internal dipole characterized by the separation of a positive charge and a negative charge on sulfur and oxygen atoms, respectively (Figure 3D). In contrast to C112, the dipolar character of C112SNO may result in the formation of an intramolecular network of hydrogen bonding and/or ion pairing with the charged residues R61 and E109 in an energetically favorable manner (Figure 3E). Consequently, such intramolecular interactions on the surface of the RB domain aided by the *S*-nitrosylation of C112 may lead to the formation of a kink in $\alpha 3$ helix coupled with further re-arrangement of $\alpha 2$ and $\alpha 3$ helices relative to each other (Figures 3B–C). Such protein conformational changes are likely to lead to distortion of the protein surface and thus may be sufficient to mitigate or completely abrogate the binding of the RB domain to the LDL receptor and thereby could provide the molecular basis of the phenotype observed upon *S*-nitrosylation of ApoE3. Alternatively, *S*-nitrosylation of C112 may also lead to change in the specificity of ApoE2 for LDL receptor substrates. It is noteworthy that R61 and E109 may also enhance the reactivity of C112 toward *S*-nitrosylation through electrostatic polarization of the thiol moiety (14).

The foregoing argument is further supported by the fact that the R158C genetic mutation in ApoE3 so as to generate the ApoE2 variant results in defective binding of the latter to the LDL receptors (16). Such loss of binding results from the disruption of a tight network of ion pairs on the surface of the RB domain of ApoE2 leading to protein conformational changes as a result of R158C mutation. Accordingly, unlike C112 being the sole site of *S*-nitrosylation in ApoE3, ApoE2 contains an additional site of *S*-nitrosylation at C158. Thus, while ApoE2 already displays defective binding toward LDL receptor, *S*-nitrosylation at both C112 and C158 will induce unfavorable protein structural changes that will further compromise such binding. In a manner akin to the close proximity of C112 to R61 and E109, our 3D structural modeling suggests that C158 lies within hydrogen bonding and/or ion pairing distance of R92 and E96 (data not shown). Thus, a scenario similar to that discussed above for intramolecular contacts aided by *S*-nitrosylation of C112 could also

exist for the *S*-nitrosylation of C158 and the effect of *S*-nitrosylation on ApoE2 structure may be even more severe than that envisioned for *S*-nitrosylation of ApoE3. It is also noteworthy that the C112R genetic mutation in ApoE3 so as to generate the ApoE4 isoform does not abrogate the binding of the latter to LDL receptors but rather changes the LDL substrate specificity (preferring VLDL over HDL) (17). Consistent with our atomic models above (Figures 3B–C), we believe that R112 may form an ion pair with E109 leading to subtle conformational changes that may change its receptor specificity but the availability of R61 could still account for its binding to LDL receptors. Overall, our atomic models presented above suggest that the *S*-nitrosylation of ApoE3 likely results in protein conformational changes that likely abrogate its binding to LDL receptors and/or change its substrate specificity. However, it is important to note that it has not been possible to directly measure the binding affinities of various ApoE isoforms to LDL receptors to further support our atomic models. Our future efforts will be directed toward this goal and to further dissect the role of NOS1-mediated *S*-nitrosylation on the function of ApoE2 and ApoE3 proteins.

In conclusion, *S*-nitrosylation has been shown to play important roles in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (18–20). Our data suggest that ApoE3 is subject to *S*-nitrosylation by NOS1 and that such post-translational modification may be involved in regulating lipid metabolism and the pathogenesis of LOAD.

Acknowledgments

We are extremely grateful to Dr. Margaret A. Pericak-Vance for providing LOAD-affected brain tissues and biotin-switch reagents. We thank Drs. Stephan Züchner and Michael Slifer for helpful discussions and critical reading of the manuscript. This work was partly supported by an RRIA grant from the Michael J. Fox Foundation for Parkinson's disease Research (to G.W.) and by funds from the National Institutes of Health (Grant# R01-GM083897) and the USylvester Braman Family Breast Cancer Institute (to A.F.)

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Figure 1. Protein interaction between ApoE isoforms and NOS1. (A) Co-IP of overexpressed ApoE isoforms and NOS1 in HEK-293 cells. (B) Co-localization of ApoE3 and NOS1 in human hippocampus by immunofluorescence.

Figure 2. S-nitrosylation of ApoE proteins. (A) Biotin-switch assay of recombinant human ApoE isoforms. (B) Biotin-switch assay of human hippocampus lysate.

Figure 3.

Effect of *S*-nitrosylation on the 3D structure of human ApoE3. (A) Fully processed ApoE3, without the N-terminal signal peptide sequence (18 residues), is comprised of an N-terminal LDL receptor binding (RB) domain and a C-terminal lipid binding (LB) domain. Note that all the amino acid numbering referred to herein is based on the amino acid sequence of the fully processed ApoE (residues 1–299). (B) 3D atomic model of the wildtype (WT) RB domain of ApoE. (C) 3D atomic model of the *S*-nitrosothiol derivative (C112SNO) of the RB domain of ApoE. Note that in both (b) and (c), the RB domains are colored brown, while the sidechain moieties of R61, E109 and C112/C112SNO are respectively colored blue, red and green. Insets show close-up views of intramolecular interactions of C112/C112SNO with R61 and E109. (D) A schematic showing the *S*-nitrosylation of C112 within the RB domain of ApoE. Note that the resulting C112SNO *S*-nitrosothiol derivative may undergo resonance arrangement to form a zwitterion with an internal dipole characterized by the separation of a positive charge and a negative charge on sulfur and oxygen atoms, respectively. (E) A schematic showing a plausible hydrogen bonding and/or ion pairing network between the polarized *S*-nitrosothiol moiety of C112SNO, the guanidino group of R61 and the sidechain carboxylate of E109. The double-headed red arrows indicate potential hydrogen bonding and/or ion pairing contacts.